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ORGAN TOXICITY AND MECHANISMS

Dectin-1 and inflammation-associated gene transcription and expression in mouse lungs by a toxic (1,3)-β-D glucan

Thomas. G. Rand · M. Sun · A. Gilyan · J. Downey · J. D. Miller

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Abstract The form of (1-3)-β-D glucan found in the cell walls of the anamorphic Trichocomaceae that grow on damp building materials is considered to have potent toxic and inflammatory effects on cells of the respiratory system. It is also considered to have a potential role in the development of non-allergenic respiratory health effects. While human studies involving experimental exposures all point to the inflammatory potential of pure curdlan, a linear (1-3)-β-D glucan in a triple helix configuration, animal experiments result in conflicting conclusions concerning the inflammatory potency of this glucan. However, because mice appear to be a better model than guinea pigs for exploring the respiratory effects of curdlan and because molecular mechanisms associated with this glucan remain largely unknown, we conducted further work to clarify the role of curdlan on the inflammatory response using our mouse model of lung disease. This study used in situ hybridization (ISH) to probe dectin-1 mRNA transcription with a digoxigenin-labeled cDNA probe, with reverse transcription (RT)-PCR based arrays used to measure inflammation gene and receptor transcriptional responses. Also, immunohistochemistry (IHC) was used to probe dectin-1 as well as anti-mouse *Ccl3, Il1-alpha,* and *TNF-alpha* expression to evaluate dose and time-course (4 and 12 h) postexposure (PE) response patterns in the lungs of intratracheally instilled mice exposed to a single 50 μl dose of curdlan at 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} M/animal (=4 μg to 4 ng curdlan/kg lung wt). Dectin-1 mRNA transcription and expression was observed in bronchiolar epithelium, alveolar macrophages (AMs), and alveolar type II cells (ATIIs) of lungs exposed to 4 μg to 40 ng curdlan/kg lung wt, at both time points. Compared to controls, array analysis revealed that 54 of 83 genes assayed were significantly modulated by curdlan. mRNA transcription patterns showed both dose and time dependency, with highest transcription levels in 10^{-7} and 10^{-8} M treatment animals, especially at 4-h PE. Nine gene mRNA transcripts (*Ccl3, Ccl11, Ccl17, Ifng, Il1α, Il-20, TNF-α, Tnfrsf1b,* and *CD40lg*) were significantly expressed at all doses suggesting they may have a central role in immunomodulating curdlan exposures. IHC revealed *Ccl3, Il1-alpha, and TNF-alpha* expression in bronchiolar epithelium, AMs and ATIIs illustrate the important immunomodulatory role that these cells have in the recognition of, and response to glucan. Collectively, these results confirm the inflammatory nature of curdlan and demonstrate the complex of inflammation-associated gene responses induced by (1-3)-β-D glucan in triple helical forms. These observations also provide a biological basis for the irritant and inflammatory response to curdlan observed in humans and animals in experimental studies.

Keywords Trichocomaceae · Molds · Damp building materials · (1-3)-β-D glucan · Curdlan · Mouse lungs · Inflammation-associated genes · RT–PCR arrays · Dectin-1 · In situ hybridization · Immunohistochemistry

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Introduction

It is well understood that respiratory health problems associated with mold exposures can be linked to both

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allergenic- and non-allergenic-respiratory responses (Health Canada 2004; National Academy of Sciences 2004) including non-atopic asthma (e.g., Cox-Ganser et al. 2005). While mechanisms accounting for atopic effects are broadly understood, those associated with non-allergenic effects are less clear. Nevertheless, there is increasing evidence that the non-allergenic effects are linked to exposures to spore and hyphal fragments from a consortium of toxin-producing, anamorphic Trichocomaceae (e.g., *Aspergillus, Eurotium, Penicillium,* and related hyphomycetes) that grow on damp building materials. These contain allergens, (1-3)-β-D glucan and low-molecular weight compounds that are either cytotoxic or have some other toxic effects, including inflammatory properties.

(1-3)-β-D Glucan is a major structural component that provides mechanical strength to the cell walls of fungi. The main route of exposure for persons living or working in damp building environments to (1-3)-β-D glucan is through inhalation of aerosolized mold spores and fungal fragments (Health Canada 2004). Studies have revealed that airborne (1-3)-β-D glucan concentrations in buildings generally range from 0.04 to 20.55 ng/m^3 (Foto et al. 2004; Reponen et al. 2007; Salares et al. 2009), but levels as high as 100 ng/m^3 have been reported (Rylander et al. 1992). The most recent studies have also revealed that the majority (70–90%) of the airborne glucan is represented by fungal particles $\leq$1.0 μm diameter, not spores (Foto et al. 2004; Reponen et al. 2007; Salares et al. 2009). The toxicological importance of these fragments on respiratory health remains to be clarified (Reponen et al. 2007). However, based on particle modeling studies (Kleinstreuer et al. 2007, 2008; Phalen et al. 2008), these fungal particles comprising the majority of (1-3)-β-D glucan in air are more efficiently deposited along respiratory bronchiolar and alveolar surfaces than intact spores. Fungal particle deposition at these lung sites is likely to have an important role in the development of non-allergenic inflammatory respiratory health effects by stimulating the glucan receptor *dectin-1* on the surfaces of immunosentinel cells (e.g., monocytes, neutrophils, mast cells, dendritic cells, and alveolar macrophages (AMs) (Douwes 2005a, b; Palma et al. 2006; Yang and Marshall 2009; Young et al. 2001, 2003a, b). This induces release of pro-inflammatory cytokines, including TNF-α, and mediates cellular responses associated with inflammation onset (Ferwerda et al. 2008). However, whether these lung surface lining cells express dectin-1 and respond similarly to glucan exposures is unknown. In the only previous study evaluating the tissue distribution of dectin-1 in situ, Reid et al. (2004) demonstrated its expression only in murine alveolar macrophages stimulated using an intraperitoneal injection with low-dose lipopolysaccharide (LPS). Whether bronchiolar and alveolar epithelial cells which are also able to function as resident innate immune cells (Kelly et al. 2008) express dectin-1 following exposure to (1-3)-β-D glucan is unclear and deserves further study.

There are a number of studies that have focused on the potential effects of (1-3)-β-D glucan on respiratory health. Results of in vitro and in vivo studies employing zymosan (a mixture of (1-3)-β-D and (1-6)-β-D glucans (1:1) in triple helical form, Ohno et al. 2001) derived from *Saccharomyces cervisiae* have demonstrated that this glucan is potently pro-inflammatory (Brown and Gordon 2001; Vassallo et al. 2000) and exhibit acute pulmonary toxicity (Young et al. 2001, 2003a, b, 2006). However, results of studies using curdlan, a pure linear (1-3)-β-D glucan (161 kDa) in triple helix configuration from *Alcaligenes faecalis,* a widely used model to study the pulmonary effects of β glucans are less clear. In vitro studies demonstrate that curdlan is a potent immunostimulant. It has been shown to activate iNos, Tnf-α, and MIP-2 expression in RAW-264.7 and RAW-R12 cell lines (Kataoka et al. 2002); complement, superoxide anion, and leukotriene B4 production in primary alveolar macrophages (Sorenson et al. 1998); Tnf-α expression in human peripheral blood mononuclear cells (PBMCs) and monocyte-derived macrophages (Ferwerda et al. 2008); and IgE-mediated histamine release from human leukocytes (Holck et al. 2007). Experimental studies in humans exposed to inhaled or nasally instilled curdlan have demonstrated that exposures can induce nasal and airway inflammatory reactions (Bonlokke et al. 2006; Sigsgaard et al. 2000; Rylander 1993, 1996). However, experimental studies in animals report conflicting results concerning the inflammatory potency of curdlan. Curdlan inhalation studies using guinea pigs have demonstrated that this glucan is only modestly inflammatory as evidenced by a small but significant increase in neutrophils in bronchioalveolar lavage fluid (BALF) and an increased infiltration of eosinophils into the airway epithelium (Sjostrand and Rylander 1997). Experiments employing guinea pigs exposed to curdlan-spiked-dust via inhalation exhibited only delayed subacute nasal congestion but no apparent respiratory or inflammatory responses (Straszek et al. 2007). Contrarily, mice intratracheally exposed to curdlan exhibited dose-dependent-like histopathological responses characterized by differences in the abundance of alveolar macrophage (AMs) and other mononuclear cells in peri-bronchiolar and adjacent intra-alveolar spaces of treatment animals after a 4 day exposure (Schuyler et al. (1998). These differences in experimental outcome of these experiments involving guinea pigs and mice may be due to the use of different animals, exposure routes, doses, and purity of the curdlan used. However, they also suggest that mice may be a better model of lung disease than guinea pigs for exploring the respiratory effects of this glucan. Because molecular mechanisms underscoring inflammatory responses in mouse lungs intratracheally instilled with

curdlan is far from fully understood, we decided that further work was needed to clarify the role of curdlan on the inflammatory response using our mouse model of lung disease.

As curdlan is a known ligand for the β-glucan receptor dectin-1 in mice (Ferwerda et al. 2008) and known to be expressed in mouse alveolar macrophages in situ (Reid et al. 2004), we hypothesized that pure curdlan exposures would result in dectin-1 transcription and expression in AMs and bronchiolar and alveolar wall surfaces exposed to this pure (1-3)-β-D glucan, resulting in dose and time-dependent inflammatory gene transcriptional and expression responses.

The objectives of this study were (1) to determine whether intratracheal curdlan exposures result in *dectin-1* transcription and expression in mouse lungs intratracheally instilled with curdlan using in situ hybridization and immunohistochemistry, respectively; (2) to evaluate dose–response and time-course profiles associated with pro-inflammatory gene transcriptional responses using mouse inflammatory gene and receptor arrays and reverse transcription (RT)-PCR to identify transcriptional signals that promote inflammatory cell recruitment and activation; and (3) to determine whether any of the genes identified as having a central role in immunomodulating curdlan exposures are expressed in cells stimulated by dectin-1.

Materials and methods

Glucan preparation

Curdlan from *Alcaligenes faecalis* (Sigma–Aldrich C7821, lot # 89H4032 ≥99% purity), which was chemically characterized by Foto et al. (2005), was used in the experiments. A fresh curdlan stock solution was prepared for each in vivo experiment by dissolving it in 0.3 M sodium hydroxide (NaOH) followed by dilution in pyrogen-free PBS to 10^{-6} M.

Animals

All mice used in the experiments were housed according to the standards of the Canadian Council for Animal Care (CCAC 1993) and with approval from the Dalhousie University Animal Care Committee. The mice were given food and water ad libitum and acclimatized for 1 week prior to use.

In vivo experiments

A total of 50 random-bred, pathogen-free Carworth Farms white (CFW) Swiss Webster 21- to 28-day-old male mice (23 ± 1.2 g (n = 25)) were divided into 2 treatment groups (control and glucan treatment). The glucan treatment animals were separated into 8 groups of 5 mice each. To test for dose-dependent effects, treatment mice were intratracheally instilled with a single 50-μl dose of curdlan at 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} M/animal (=4 μg to 4 ng curdlan/kg lung wt) (mean lung wt = 0.25 ± 0.028 g (n = 25). At each dose used, time course of effects was studied at 4- and 12-h postinstillation (PI). Two groups of 5 control animals each were intratracheally instilled with carrier saline. The selection of the doses employed in these experiments was based on $PM_{2.5}$ dosimetry for resting and moderate nasal breathing and for moderate oral breathing, i.e., all particulate matter ≤2.5 μm in size, which based on (Foto et al. 2004; Brown et al. 2005) comprises 70–90% of the total airborne β-glucan. On a 24 h basis, lung exposure to β-glucan in spore and hyphal fragments <2.5 μm for resting and moderate nasal breathing, and moderate oral breathing, would be in the order of 10^{-7}–10^{-9} M, focused on a number of 'hot spots' (Phalen et al. 2008).

Before instillation, mice were lightly anaesthetized with an intraperitoneal injection of 0.3 ml of an anesthetic mixture consisting of 30% ketamine (Ketaleen® (100 mg/ml)), 5.2% xylazine (Rompun® (100 mg/ml), and 64.8% physiological saline. Once anaesthetized, each mouse was placed on an intratracheal instillation board 20° from the vertical as described in Mason et al. (1998) and instilled with 50 μl of glucan, as described earlier. Control animals were instilled with 50 μl of carrier saline only. Mice were left in the upright position on the instillation board for approximately 2 min before being placed back in their cages on a warm pad to recover. During recovery, mice were monitored for signs of sickness or distress as outlined in CCAC guidelines (CCAC 1993).

Treatment and control animals were killed 5 at a time at 4 and 12 h PI using a 0.3 ml IP injection of Euthanol® (sodium pentobarbital, 50 mg/ml). The mice were immediately exsanguinated by cutting the abdominal artery, and the lungs were removed. The right lung was excised and immediately immersed in 5 ml of cold RNAlater® then gently teased with forceps to remove the major respiratory tree components (i.e., trachea, bronchus, and large bronchioli) after Mason et al. (2001). The remaining tissues were suspended in 1-ml RNAlater® and stored at −20°C for later study. The left lung was excised and immediately fixed in fresh 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) pH 7.3, prepared using diethylpyrocarbonate water (DEPC-water), for 6 h, for in situ hybridization and immunohistochemistry, as described in the following paragraph.

In situ hybridization (ISH)

To determine whether curdlan exposures would stimulate dectin-1 gene transcription, ISH was employed to detect

and localize expression in mouse lungs intratracheally instilled with 10^{-7}–10^{-10} M curdlan.

Preparation of RNA probes

Total RNA extraction

For ISH, AMs were collected from the bronchioalveolar lavage fluid (BALF) of 7- to 8-week-old mice killed using a pentabarbitol overdose. Mice were lavaged with 5 ml of a cold (4°C) solution of 0.1 g of lidocaine (Sigma–Aldrich) in 50 ml of pyrogenic-free PBS, after Mason et al. (1998). The lavage samples were centrifuged at 300 rcf for 10 min at 4°C. The AMs were pelleted, the supernatant removed, and cell aliquots examined microscopically for cell purity (±94%) and enumeration. Cells were cultured in RPMI 1× Medium 1640 (Gibco) supplemented with 5% fetal bovine serum (Sigma–Aldrich), 1% of antibiotic/antimycotic (penicillin-G/streptomycin; Sigma–Aldrich), and 0.015 g of L-glutamine (Sigma–Aldrich) in 24-well culture plates and incubated overnight at 95% air/5% CO_2 and 37°C. AMs were then exposed to 10^{-7} M (1-3)-β-D glucan for 1 h.

Total cellular RNA was extracted from 5×10^5 AMs. The extraction was performed using RNeasy (Qiagen) according to the manufacturer's instructions. After extraction and washing, RNA was suspended in RNase/DNase-free water (Sigma–Aldrich) and concentration and purity determined using a NanoDrop® ND-1000.

Reverse transcription

The RNA was used to make cDNA according to SuperArray's RT^2 Profiler PCR Array System, according to the manufacturer's instructions. The cDNA was then stored at −20°C until needed.

PCR amplification of cDNA

cDNA was amplified using a polymerase chain reaction (PCR) according to the manufacturer's guidelines for the Faststart Taq DNA Polymerase, dNTPack (Roche Molecular Biochemicals, Laval, PQ). The dectin-1 primers (10 µM solutions) were purchased from Integrated DNA Technologies using GeneBank sequence NM_02008 with T3 and T7 promoters on the sense (S) and antisense (AS) strands, respectively (Table 1). A 25-µl reaction was set up according to the Roche manual. Upon completion of PCR, gel electrophoresis was carried out using the PCR products on a 2% agarose gel to check the quantity and for quality assurance (data not shown).

The PCR products were cleaned using Millipore's Montage PCR® to remove excess primer and dNTPs. Following phenol/chloroform extraction (Sambrook et al. 1989) to remove excess proteins, PCR products were centrifuged for 5 min at 15,000×*g* and the supernatant precipitated with 7.5 M ammonium acetate (DEPC treated) and 100% ethanol. The pellet was then resuspended in 15-µl RNase-free water with concentration and integrity of cDNA in the samples determined using a NanoDrop® ND-1000.

Probe generation

Digoxigenin-labeled probes were produced using the Digoxigenin-UTP in vitro transcription kit (Roche Molecular Biochemicals, Laval, PQ) according to the manufacturer's suggested protocol, with modest modification outlined in Murray et al. (2003). To check probe integrity, a RNA dot blot was performed after Murray et al. (2003) on both the AS and S strands using Hybond XL membranes (data not shown). Serial dilutions of the cDNA probe template were prepared at 50, 5, 0.5, and 0.05 ng. The probes were diluted to 1:50 solution with hybridization buffer and added to the membranes; the membranes were then sealed in a plastic bag and baked at 60°C for 4 h for hybridization. After hybridization, the membranes were washed in 2× SSPE/0.5% SDS for 15 min, followed by 0.1× SSPE/0.5% SDS for 1 h at 60°C. After final rinses in Tris–HCl (pH 7.5) 2× for 5 min each, Roche anti-digoxigenin-AP, Fab-fragments were diluted to a 1:500 solution, added to the membrane, and incubated for 30 min at 40°C. The membranes were then rinsed 3× in Tris–HCl (pH 7.5), for 5 min each, followed by a wash in Tris–HCl (pH 9.5) for 10 min. The membranes were then incubated in the chromogenic solution 4-nitro blue tetrazolium chloride (NBT); 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) in the dark until the spots became visible. The membrane was then rinsed in Tris–HCl (pH 7.5) and dehydrated in an ascending ETOH series.

Table 1 Oligonucleotide primers and their sequences with respective RNA polymerase promoters and sequences

Primer	Nucleotide sequence (5′⇒3′)	RNA polymerase	Promoter sequence[a]
Dectin 1 forward	ATAGGCCTTTCCCGCAATCAGAGT	T3	AATTAACCCTCACTAAAGGGA
Dectin 1 reverse	TCTGTTGTTGGTAGTGGTGGTGGT	T7	TAATACGACTCACTATAGGGA

[a] The promoter sequences were added to the 5′ end of primer sequences

Hybridization

For the hydridization experiments, 4% paraformaldehyde-fixed carrier control and treatment (4 μg to 4 ng curdlan/kg lung wt) left lung tissues from 4 animals per group were dehydrated through an ascending ethanol series, cleared in xylene, and embedded in Paraplast Plus®. Paraffin-embedded lung tissues were sectioned at 6 μm thick and mounted on 3-aminopropyltriethoxysaline (TESPA)/DEPC-treated slides (Jowett 1997). Sections were dewaxed, hydrated through a descending ETOH series, washed in DEPC-water then predigested using 2-μl proteinase K (2 mg/ml) in 20 ml of 50 mM Tris–HCl for 30 min at 37°C. Following digestion, slides were rinsed in 2 mg/ml of glycine for 2 min. They were then treated with 0.1 M triethanolamine (TEA) for 5 min, then 0.1 M TEA with 62.5-μl acetic acid for 10 min, followed by 0.1 M TEA for 5 min. For each probe, ISH assays were performed on two sets of three slides from the same block, after Murray et al. (2003). One set was assayed using the AS probe and one with the S probe, both diluted 1:2 with in situ hybridization buffer. Fifty microliters of the probe was then added to each slide. Hybridization was allowed to proceed overnight in a closed humid incubation chamber lined on the bottom with blotting paper infiltrated with 50% formamide, 2× SSC at 47°C. Following hybridization, sections were rinsed in 2× SSC for 5 min ea. at room temperature. They were then incubated for 1 h in 50% formamide, 2× SSC at 45°C, rinsed in buffer (10 mM Tris, pH 8.0, and 500 mM NaCl) at room temperature for 5 min and further incubated for 30 min at 37°C in the same buffer amended with 40 μl of 10 mg/ml RNase A and 2 μl of 100,000 U/ml RNase T1. Sections were rinsed in unamended buffer for 30 min at 37°C then rinsed 3× in 2× SSC, 1× SSC, and 0.5× SSC for 30 min at 47°C ea.

For digoxigenin detection, slides were incubated in 100 μl of a 1:250 sheep anti-DIG phosphatase-conjugated antibodies (Roche Applied Science) following a 30-min block in 0.1 M Tris–HCl, pH 7.5 150 mM NaCl containing 10% lambs normal serum and 1% BSA (Sigma–Aldrich) for 30 min at 37°C in a humid chamber. Following antibody treatment, the slides were washed in blocking buffer without lamb serum by gentle rocking for 30 min. After the antibody wash, slides were then rinsed 3 times, for 10 min ea. in 100 ml of buffer I (0.1 M Tris–HCl, pH 9.5), 1 g of 1% BSA, and 0.1 ml of 0.1% Tween 20) followed by a single rinse in buffer II (0.1 M Tris–HCl, 0.1 M NaCl, and 50 mM $MgCl_2$) for 10 min. To develop a color reaction, slides were placed in chromagen solution and processed after Rand and Miller (2008).

Immunohistochemistry

All immunohistochemical localization experiments were performed using the same 4% paraformaldehyde-fixed lung tissue blocks employed for ISH. Immunohistochemical localization of dectin-1 was performed using procedures outlined in Gregory et al. (2004), with modest exception. Briefly, 6-μm thick lung sections, on TESPA-coated glass slides were assigned to control or test groups. Sections were either immunolabeled or used as control for blocking by employing a heat-induced epitope retrieval (HIER) protocol (Happerfield et al. 1996). Sections were dewaxed then hydrated through a descending ETOH series, washed in 0.05 M Tris–HCl buffer (pH 7.6), placed in boiling citrate buffer (pH 5.8) for 10 min and then allowed to cool at room temperature for 20 min. They were rinsed 3× in TBS (pH 7.8), 4 min each. Slides were placed in 50 ml of 0.5% trypsin solution and incubated in a water bath at 37°C for 15 min, then rinsed 2× in Tris–HCl pH 7.8, 4 min. each. After rinsing, slides were washed 2× in phosphate buffer saline (PBS) pH 7.6, 4 min each, then blocked using 50 ml of 10% lamb serum in 0.01 M PBS, at room temperature, for 30 min. After blocking, test slides were exposed to 70 μl of 1:75 goat anti-mouse dectin1/CLEC7A polyclonal antibody (R&D) in PBS (pH 7.6) solution while control slides were exposed only to PBS. Both test and control slides were incubated in a humid chamber overnight at 4°C. After incubation, slides were rinsed 2× in PBS (pH 7.6) for 4 min each followed by rinsing 2× in Tris–HCl (pH 7.6) for 4 min. each. After rinsing, slides were covered with 70 μl of a 1:1000 anti-goat-BIOT: Tris–HCl (pH 7.5) and incubated in a humid chamber at room temperature for 1 h. After incubation, sections were rinsed 3× in Tris–HCl (pH 7.6), 4 min each. They were then exposed to anti-biotin-alkaline phosphatase, Fab-fragments (AB Roche Molecular Biochemicals) at a working concentration of the 1:1000 3^0 antibody: Tris–HCl (pH 7.6) for 1 h then rinsed 3× in Tris–HCl (pH 8.0), 4 min each and then in buffer II (=100 ml Tris–Base (pH 9.5); 0.580 g NaCl; 1.015 g $MgCl_2$) solution at room temperature for 10 min. To develop a color reaction, slides were placed in the chromagen solution and processed, as described earlier.

RNA extraction and reverse transcription real-time PCR

Total RNA isolation was performed using Trizol® reagent (Gibco BRL) according to the manufacturer's specifications. Briefly, right lung tissues were homogenized in 1 ml of Trizol® reagent using a CAT ×120 electric homogenizer (Rose Scientific Ltd, Edmonton AB). After addition of chloroform and centrifugation at 4°C, the aqueous layer was separated and RNA was precipitated with isopropanol. The precipitated RNA solution was again centrifuged at 4°C to pellet the RNA. The RNA pellet was washed with 75% ETOH and resuspended in RNAse-free water (Sigma–Aldrich). The concentration of RNA in samples was determined using a NanoDrop® ND-1000. RNA integrity and purity was assessed using RNA Nano chips (Agilent 2100

Bioanalyzer). Samples with 260/280 nm ratio of ≥2.0 were used for array analysis.

Reverse transcription real-time PCR and array analysis

Reverse transcription PCRs were carried out using a reaction ready first strand cDNA synthesis kit (SuperArray, Bioscience Corp.) according to manufacturer's instructions. The reverse transcription and real-time PCRs were carried out using 96-well PCR arrays designed for the evaluation of mouse inflammatory genes and receptors (# PAMM-011 SuperArray Bioscience Corp®) and using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Relative gene expression was determined according to the comparative C_t method, with the β-actin (Actb) housekeeping gene and negative control references for DNA contamination set as the calibrators. Fold change equals $2^{\Delta\Delta Ct}$, where the C_t is the threshold cycle, ΔC_t is the difference between the C_t values of the target gene and the internal control gene, $\Delta\Delta C_t$ represents the difference between the ΔC_t value for the control and treated lung tissues.

Statistical analysis

Genes and treatment groups were clustered using the SuperArray web-based hierarchical clustering software package for the PCR Array System (http://www.sabiosciences.com/pcrarraydataanalysis.php). A Shapiro–Wilks test for normality was performed to verify if the samples were normally distributed. A two-tailed unpaired *t*-test ($P \leq 0.05$) was then performed to determine if the treatments showed significant inflammatory gene expression in comparison with the saline controls. All data are shown as their mean fold change values. One-way ANOVA and Tukey's pair-wise analyses were also carried out using SYSTAT version 11.0. Gene transcription results were considered significant if they had ≥1.5-fold or ≤−1.5-fold change at $P \leq 0.05$ probability level.

Induction of *Ccl3, IL1a*, and *Tnf-α* expression

To confirm gene array data that cytokine gene transcription was being translated to cytokine protein expression in treatment lungs, immunohistochemistry was used to detect *Ccl3, IL1a*, and *Tnf-α* expression in 4% paraformaldehyde-fixed lung tissues. These cytokines were determined to be among the few transcribed in animals exposed to all curdlan doses. Their detection in situ was accomplished using procedures outlined earlier, with the following modest exception. After blocking, test slides were exposed to either goat IgG anti-mouse IL1α BIOT Fab-fragments 1:100 (R&D Systems), goat IgG anti-mouse Ccl3/MIP-1-BIOT Fab-fragments 1:75 (R&D Systems), or goat IgG anti-mouse Tnf-α-BIOT Fab-fragments 1:75 (R&D Systems) in 0.01 M PBS (pH 7.6) solution while negative control slides were exposed only to PBS. A positive control tissue was mouse skin that exhibits positive staining results with TNF-α (ICH World). Test and control slides were incubated in a humid chamber at 37°C for 2 h. After incubation, slides were rinsed 2× in PBS (pH 7.6), 4 min each followed by rinsing 2× in Tris–HCl (pH 7.6) at 4 min each. After rinsing, slides were covered with 70 µl of a 1:250 anti-biotin-alkaline phosphatase, Fab-fragments (AB Roche Molecular Biochemicals) in 0.05 M Tris–HCL (pH 7.8) and incubated in a humid chamber at 37°C for 45 min. After incubation, slides were rinsed 3× in Tris–HCl (pH 7.6), 4 min each and then in buffer II (=100 ml Tris–Base (pH 9.5); 0.580 g NaCl; 1.015 g $MgCl_2$) solution at room temperature for 10 min. After color reaction and color development (described earlier), slides were rinsed 3× in Tris–HCl (pH 8.0) buffer solution, 4 min each, dehydrated in an ascending ETOH series and mounted using Hydromatrix®.

All imaging was accomplished using a Leica DMRE microscope with Simple PCI image capture system (Compix, C-Imaging Systems, Cranberry Township, PA, USA).

Results

None of the control or treatment animals used in the experiments showed signs of illness or respiratory distress due to instillation procedures.

In situ hybridization

Using AS RNA probe to the *dectin-1* gene transcript, a light colorimetric reaction was obtained in respiratory epithelia in both 4- and 12-h treatment animals (Fig. 1b, d) compared to controls (Fig. 1a, c). In animals exposed to 4 µg to 40 ng curdlan/kg lung wt for 4 and 12 h, AS probe hybridized to medial and apical surfaces of terminal bronchiolar epithelia (Clara cells) (Fig. 1d), and on some but not all alveolar macrophages and alveolar type II cells (Fig. 1e, f). Signal was not observed in lung sections of animals exposed to 4 ng curdlan/kg lung wt at either time point. Signal was also not observed in lung sections after hybridization with the S RNA probe.

Dectin-1 immunohistochemistry

Dectin-1 expression

Carrier saline treated lung tissues showed only modest evidence of dectin-1 expression (Fig. 2a, b). However, compared to controls, lung tissues from animals exposed to

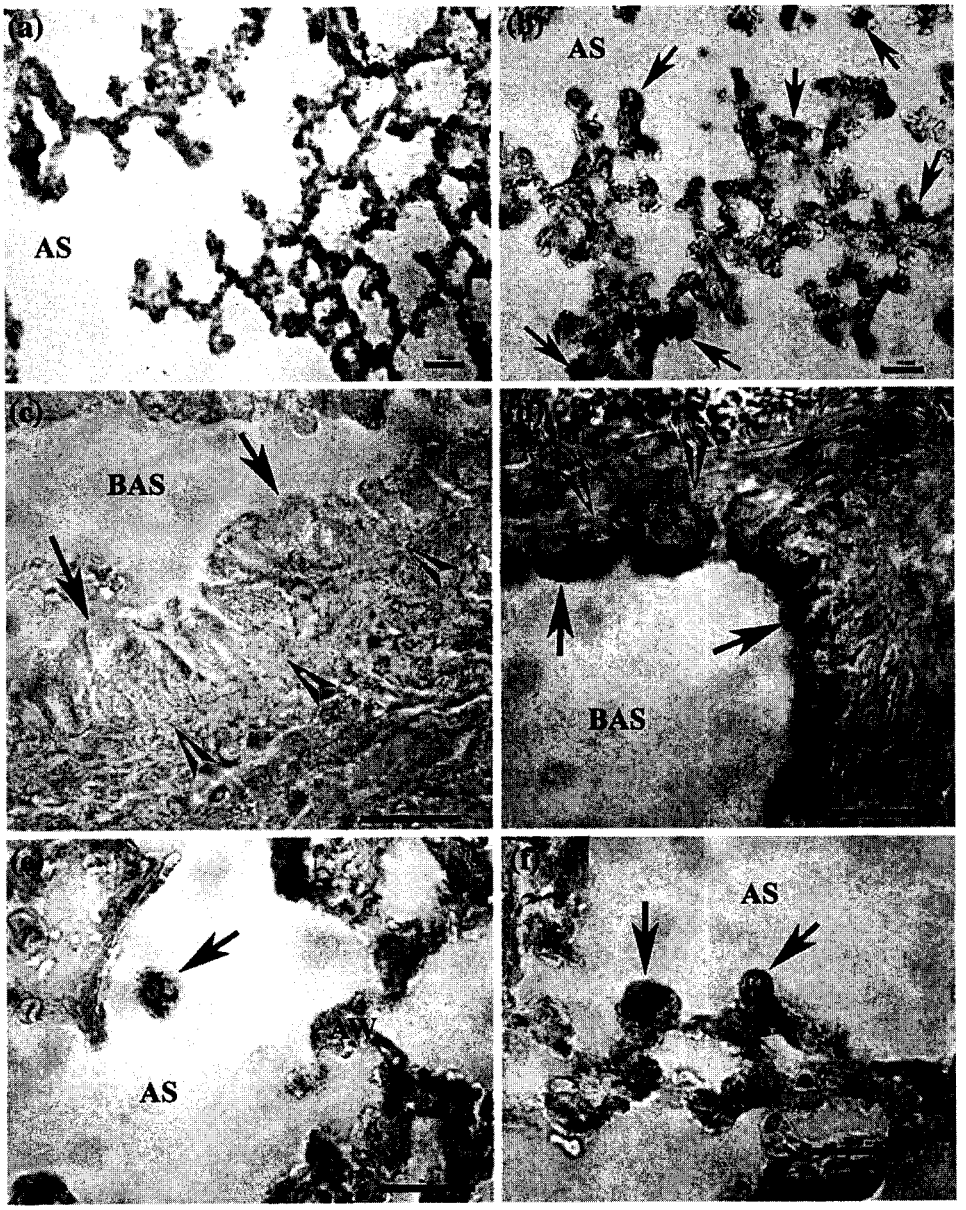

Fig. 1 **a** Dectin-1 antisense RNA probed carrier control lung at 4-h PE (AS = alveolar space) showing only modest positive staining. **b** Dectin-1 antisense RNA probed lungs exposed to 0.4 μg curdlan/kg lung wt (4-h PE) highlighting dectin-1 positive alveolar wall cells (*arrows*) (AS = alveolar space). **c** Dectin-1 antisense RNA probed carrier control terminal bronchiole at 12-h PE showing an apparent lack of positive staining. *Arrow heads* indicate epithelial basement membrane, and *arrows* indicate epithelial apices (BAS = bronchio-alveolar space). **d** Dectin-1 antisense RNA probed lungs exposed to 40 ng curdlan/kg lung wt at 12-h PE showing dectin-1 mRNA positive probing along apices and medial areas (*arrows*) of respiratory bronchiole epithelia. *Arrow heads* indicate epithelial basement membrane (BAS = alveolar space). **e** Dectin-1 antisense RNA probed lungs exposed to 40 ng curdlan/kg lung wt at 12-h PE showing dectin-1 positive alveolar macrophage (*arrow*) within alveolar space (AW = alveolar wall). **f** Dectin-1 antisense RNA probed lung exposed to 40 ng curdlan/kg lung wt at 12-h PE showing dectin-1 positive alveolar type II cells (*arrow*) (AS = alveolar space, AW = alveolar wall). *Bar scales* = 20 μm

concentrations as low as 40 ng curdlan/kg lung wt exhibited more intense positive staining with the anti-dectin-1 antibody, especially after 12-h exposure (Fig. 2c–f). Staining was heaviest along the medial to apical regions of the bronchiolar epithelia (Fig. 2c, d) and in the cytoplasm of some but not all AMs (Fig. 2e) and ATIIs (Fig. 2f).

Glucan-induced inflammation-associated gene modulation

Compared to controls, 54 of 83 inflammation-associated genes assayed were significantly modulated ($P \leq 0.05$; $\geq$1.5-fold or ≤ -1.5-fold change) in lungs of curdlan-exposed mice (Fig. 3; Table 2). Heat map profiling revealed

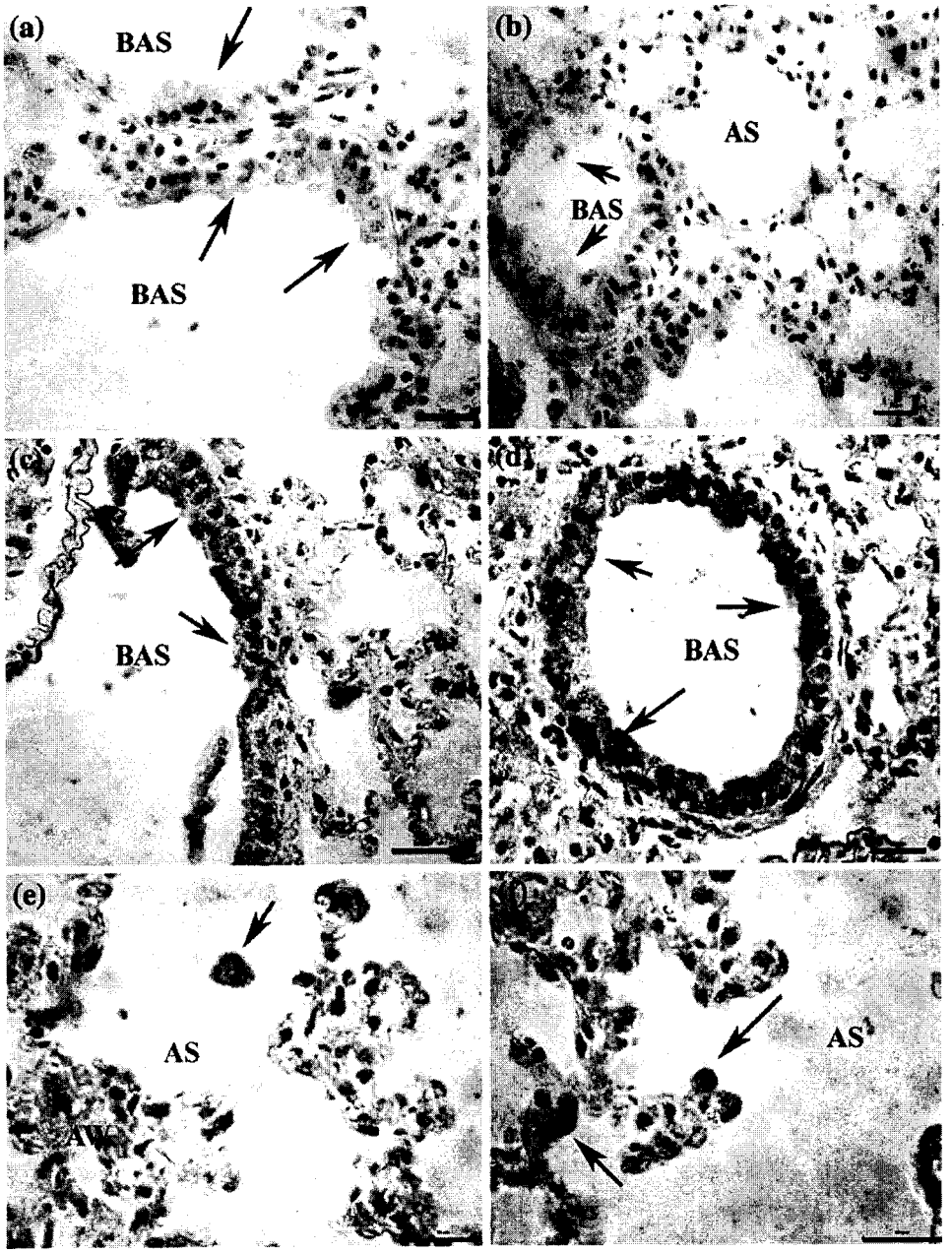

Fig. 2 **a** Anti-mouse dectin-1 immunohistochemical staining of 4-h PE carrier control animal lung showing an apparent lack of positive dectin-1 staining along bronchiolar epithelium (*arrows*). **b** Anti-mouse dectin-1 immunohistochemical staining of 12-h PE carrier control animal lung showing an apparent lack of positive dectin-1 staining along bronchiolar epithelium (*arrows*) and alveolar surfaces. **c** Anti-mouse dectin-1 immunohistochemical staining of 4-h PE treatment animal lung exposed to 40 ng curdlan/kg lung wt showing positive staining along bronchiolar epithelium (*arrows*). Anti-mouse dectin-1 immunohistochemical staining of 12-h PE treatment animal lung exposed to 40 ng curdlan/kg lung wt showing positive staining along bronchiolar epithelium (*arrows*). **e** Dectin-1 immunohistochemical staining of 12-h PE treatment animal lung exposed to 40 ng curdlan/kg lung wt showing positively stained alveolar macrophage (*arrow*) in alveolar space. **f** Dectin-1 immunohistochemical staining of 12-h PE treatment animal lung exposed to 40 ng curdlan/kg lung wt showing positively stained alveolar type II cells (*arrows*). AS = alveolar space, AW = alveolar wall; BAS = bronchio-alveolar space). *Bar scales* = 20 μm

that compared to controls, the genes most modulated were associated with the two higher dose treatments (4 μg to 0.4 μg curdlan/kg lung wt) and that they clustered into 2 groups: one group comprising up-regulated genes and a smaller group comprising down-regulated genes. Compared to these two high-dose treatments, gene transcription responses in the treatments receiving the two lowest doses of curdlan (40 ng to 4 ng curdlan/kg lung wt) were more

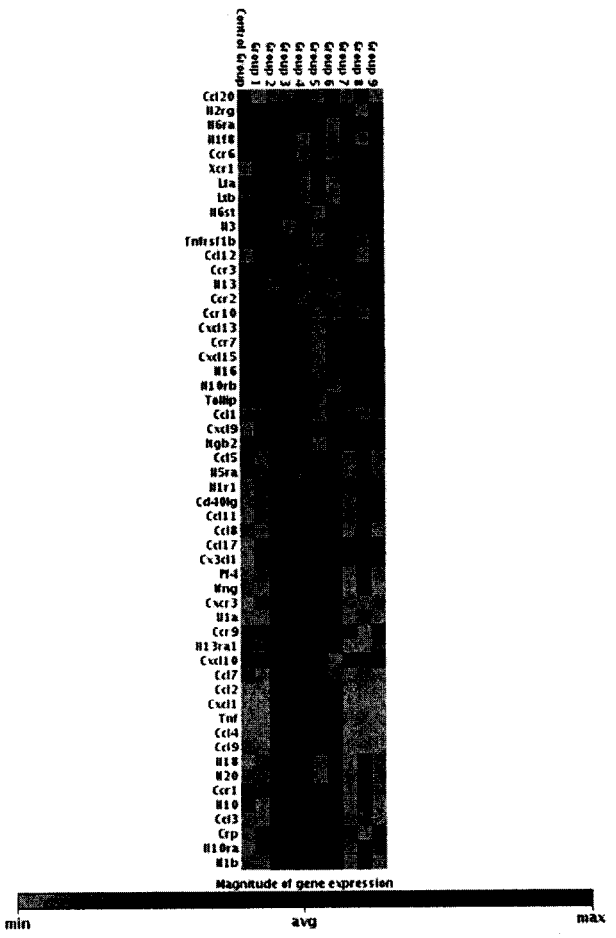

Fig. 3 Inflammation-associated gene heat map showing significant ($P \leq 0.05$; $\geq$ 1.5-fold or $\leq$ -1.5-fold change) transcription patterns in mice intratracheally instilled with 4 μg to 4 ng curdlan/kg lung wt. *Red* and green cells signify genes that were either up- or down-regulated, respectively, after treatment compared to controls. The *scale* represents fold changes (*red* up-regulated; *green* down-regulated; *black*, average change). Control animals = saline control animals 4-h PE; group 1 = saline control animals 12-h PE; group 2 = 4 μg treatment 4-h PE; group 3 = 4 μg treatment 12-h PE; group 4 = 0.4 μg treatment 4-h PE; group 5 = 0.4 μg treatment 12-h PE; group 6 = 40 ng treatment 4-h PE; group 7 = 40 ng treatment 12-h PE; group 8 = 4 ng treatment 4-h PE; group 9 = 4 ng treatment 12-h PE

similar to controls (Fig. 3). Of the 54 modulated genes, 41 were significantly up-regulated between 1.5- and 31.5-fold while the remainders were down-regulated from −1.50 to −4.3 fold. Significantly modulated genes were predominately C–C motif chemokines (12) and their receptors (7), followed by interleukins (10) and their receptors (7), C–X–C motif chemokines (7) and their receptors (2), and TNF cytokine family (3) and receptor (1) (Table 2).

The greatest number of significantly modulated genes were in lungs exposed to 4 μg curdlan/kg lung wt (54 genes) and 10^{-8} M curdlan (40 genes) while the lowest numbers were in the 40 ng curdlan/kg lung wt (21 genes) and 4 ng curdlan/kg lung wt treatment animals (9 genes). Twenty-one genes (*Ccl1, Ccl2, Ccl3, Ccl4, Ccl9, Ccl11, Ccl12, Ccl17, Ccr9, Ccr10, Crp, Cxcl1, Cxcl9, Cxcr3, Ifng, Il1α, Il20, Itgb2, Tnf-α, Tnfrsf1b,* and *CD40lg*) were significantly transcribed in animals exposed to 4 μg to 40 ng curdlan/kg lung wt while 9 genes, (*Ccl3, Ccl11, Ccl17, Ifng, Il1α, Il-20, TNF-α, Tnfrsf1b,* and *CD40lg*) were significantly transcribed at all doses. Transcription levels of these 9 individual genes in animals exposed to the different curdlan concentrations exhibited dose–response characteristics: highest transcription levels were in 4 μg curdlan/kg lung wt treatment animals; lowest levels were in 4 ng curdlan/kg lung wt treatment mice (Table 2). Some significantly modulated genes also exhibited temporal response patterns. With the exception of 4 μg curdlan/kg lung wt treatment animals in which 42 genes were transcribed at both 4- and 12-h PE, highest numbers of significantly transcribed genes were in lungs of 4-h treatment animals (Table 2). Additionally, 8 genes (*Crp, Cxcl9, Cxcl10, Cxcr3, Il10rb, Itgb2, Il-18, Il1r1, Tnfrsf1b,* and *Tollip*) were significantly up-regulated at 4-h PE only; 13 (*Ccl5, Ccr1, Ccr2, Ccr3, Ccr10, Cxcl13, Cxcl15, Il-13, Il3, IL16, Il6ra, Il2rg,* and *Il6st*) were significantly modulated in 12-h treatment mice (Table 2).

Ccl3, *IL1a,* and *Tnf-α* immunohistochemistry

Carrier control lungs showed no evidence of positive staining for anti-mouse *Ccl3*, *IL1a,* and *Tnf-α* antibodies (Fig. 4a–c). However, positive *Ccl3*, *IL1a,* and *Tnf-α* staining was observed in animals exposed to doses as low as 40 ng curdlan/kg lung wt. Medial to apical regions of bronchiolar epithelium stained positively for these cytokines, although staining was less intense at 4-h PE than at 12-h PE (Fig. 4d–i). In addition to bronchiolar epithelial staining, ATIIs and AMs were also stained with all three antibodies (Fig. 5a–f). Mouse skin positive control showed heavy staining while control sections were unstained for anti-*Tnf-α* antibodies (data not shown).

Discussion

The results of this study support the position that curdlan potentiates pulmonary pro-inflammatory responses in mice. Curdlan was chosen because the sole reliable experimental data for humans was obtained with curdlan and because of its similarity with zymosan in activating the *Limulus* glucan receptor (Ohno et al. 2001; Tanaka et al. 1991).

Using in situ hybridization (ISH) and immunohistochemistry (IHC), our results have revealed that low-dose curdlan (40 ng curdlan/kg lung wt) induces *dectin-1* gene transcription and expression in proximal lung regions. This confirms a previous study that *dectin-1* is expressed in the

Table 2 Inflammation-associated gene changes in curdlan-exposed mouse lungs at 4- and 12-h PE

RefSeq	Gene	Gene description	4 μg		0.4 μg		40 ng		4 ng	
			4 h	12 h	4 h	12 h	4 h	12 h	4 h	12 h
NM_011329	*Ccl1*	Chemokine (C–C motif) ligand 1	6.25	1.83	2.44	1.63	1.7			
NM_011330	*Ccl11*	Small chemokine (C–C motif) ligand 11	3.31	2.93	2.03	3.32	2.03		1.66	
NM_011331	*Ccl12*	Chemokine (C–C motif) ligand 12	2.6		3.22	3.32	2.29	3.17		
NM_011332	*Ccl17*	Chemokine (C–C motif) ligand 17	4	2.98	3.16	3.2	1.68		1.88	
NM_011333	*Ccl2*	Chemokine (C–C motif) ligand 2	4.34	3.68	**17.84**	6.28	2.72			
NM_016960	*Ccl20*	Chemokine (C–C motif) ligand 20	−4.3	4.9						
NM_011337	*Ccl3*	Chemokine (C–C motif) ligand 3	13.73	**29.91**	**15.16**	**20.39**	6.14		1.59	
NM_013652	*Ccl4*	Chemokine (C–C motif) ligand 4	7.96	11.45	12.12	5.81	3.43			
NM_013653	*Ccl5*	Chemokine (C–C motif) ligand 5		2.67		3.18				
NM_013654	*Ccl7*	Chemokine (C–C motif) ligand 7	2	2.15	3.66					
NM_021443	*Ccl8*	Chemokine (C–C motif) ligand 8	3.15	1.66	1.94	3.66				
NM_011338	*Ccl9*	Chemokine (C–C motif) ligand 9	2.78	4.92	3.07	2.36	1.53			
NM_009912	*Ccr1*	Chemokine (C–C motif) receptor 1		1.84		1.61				
NM_009915	*Ccr2*	Chemokine (C–C motif) receptor 2		5.05						
NM_009914	*Ccr3*	Chemokine (C–C motif) receptor 3		−1.84		−1.81				
NM_009835	*Ccr6*	Chemokine (C–C motif) receptor 6	2.46							
NM_007719	*Ccr7*	Chemokine (C–C motif) receptor 7		2.08						
NM_009913	*Ccr9*	Chemokine (C–C motif) receptor 9	1.94	1.88	1.56		1.53			
NM_007768	*Crp*	C-reactive protein, pentraxin-related	3.75		4.06		2.05			
NM_009142	*Cx3cl1*	Chemokine (C–X3–C motif) ligand 1	1.75	1.59	1.62	1.62				
NM_008176	*Cxcl1*	Chemokine (C–X–C motif) ligand 1	7.86	11.77	**24.59**	10.88	4.54			
NM_021274	*Cxcl10*	Chemokine (C–X–C motif) ligand 10	2.03		3.27	1.78				
NM_018866	*Cxcl13*	Chemokine (C–X–C motif) ligand 13		−2.67						
NM_011339	*Cxcl15*	Chemokine (C–X–C motif) ligand 15	1.18							
NM_019932	*Cxcl4*	Chemokine (C–X–C motif) ligand 4	1.64	1.54						
NM_008599	*Cxcl9*	Chemokine (C–X–C motif) ligand 9	3		1.72		1.98			
NM_009910	*Cxcr3*	Chemokine (C–X–C motif) receptor 3	1.72		1.68		1.67			
XM_894898	*Ccr10*	Chemokine (C–C motif) receptor 10		−1.59		−2.66		1.59		
NM_008337	*Ifn-λ*	Interferon gamma	3.01	3.58	2.02	2.21	1.73		1.85	
NM_010548	*Il10*	Interleukin 10	1.9	7.38	1.8	6.29				
NM_008348	*Il10ra*	Interleukin 10 receptor, alpha	1.66	1.67	1.67	1.54				
NM_008349	*Il10rb*	Interleukin 10 receptor, beta	1.58		1.54					
NM_008355	*Il13*	Interleukin 13		2.26						
NM_133990	*Il13ra1*	Interleukin 13 receptor, alpha 1	1.57	1.55						
NM_010551	*Il16*	Interleukin 16		−1.59		−1.57				
NM_008360	*Il18*	Interleukin 18	1.75		1.65					
NM_010554	*Il1a*	Interleukin 1 alpha	**19.85**	**31.54**	7.62	**16.64**	5.82	8.79	2.32	
NM_008361	*Il1b*	Interleukin 1 beta	1.87	5.3	2.49	2.23				
XM_130058	*Il1f8*	Interleukin 1 family, member 8	1.78	2.51	−2.3					
NM_008362	*Il1r1*	Interleukin 1 receptor, type I	1.61		1.55	1.64				
NM_021380	*Il20*	Interleukin 20	2.44	2.89	2.85		1.52		1.71	
NM_013563	*Il2rg*	Interleukin 2 receptor, gamma chain	1.82	1.51						
NM_010556	*Il3*	Interleukin 3		−2.66						
NM_008370	*Il5ra*	Interleukin 5 receptor, alpha	1.79	1.74		1.67				
NM_010559	*Il6ra*	Interleukin 6 receptor, alpha		1.59		1.53				
NM_010560	*Il6st*	Interleukin 6 signal transducer		−3.18		−3.28				

Table 2 continued

RefSeq	Gene	Gene description	4 μg		0.4 μg		40 ng		4 ng	
			4 h	12 h	4 h	12 h	4 h	12 h	4 h	12 h
NM_008404	*Itgb2*	Integrin beta 2	1.62		1.54		1.52			
NM_010735	*Lta*	Lymphotoxin A	2.06	2.43						
NM_008518	*Ltb*	Lymphotoxin B	2.32	1.86						
NM_013693	*Tnf*	Tumor necrosis factor	**27.75**	**23.25**	**33.24**	7.49	5.49	4.79	2.61	
NM_011610	*Tnfrsf1b*	Tumor necrosis factor receptor superfamily, member 1b	1.51	−1.57	2.33	−1.77	−1.51		−1.54	
NM_011616	*Cd40lg*	CD40 ligand	3.11	3.18	2.5	3.23	2.2		2.45	
NM_023764	*Tollip*	Toll-interacting protein	1.6		1.88					
NM_011798	*Xcr1*	Chemokine (C motif) receptor 1	2.51	1.64						

Bolded numbers indicate gene transcriptional levels ≥15-fold greater than controls

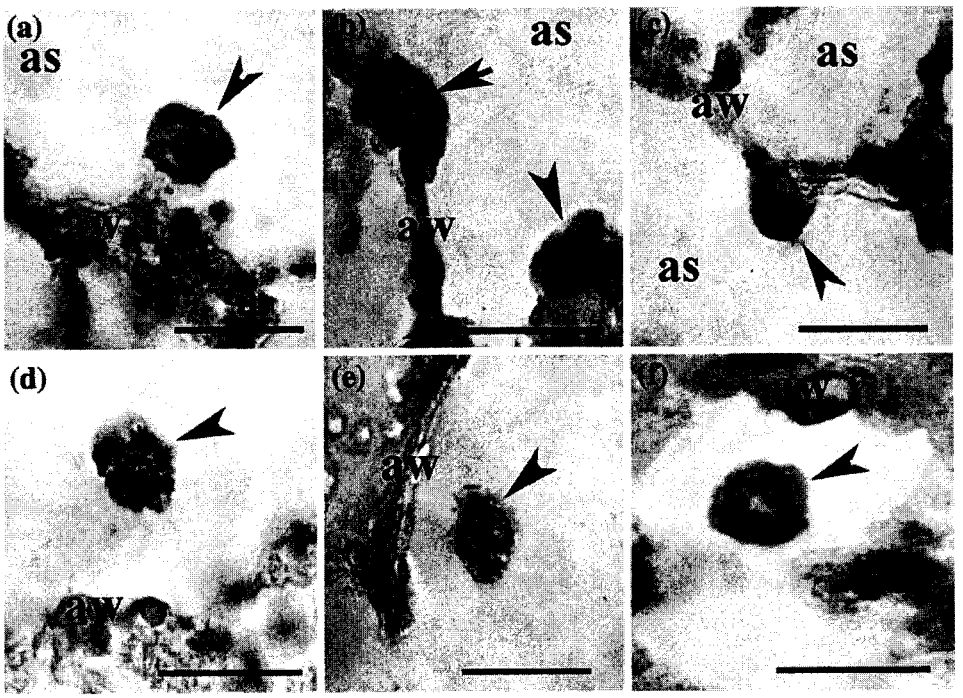

Fig. 4 **a** 12-h PE anti-mouse Ccl3 probed lungs exposed to 40 ng curdlan/kg lung wt showing weak perinuclear staining of ATII cell (*arrow*). **b** 12-h PE anti-mouse IL1α probed lungs exposed to 40 ng curdlan/kg lung wt showing weak positive staining of ATIIs (*arrows*). **c** 12-h PE anti-mouse Tnf-α probed lungs exposed to 40 ng curdlan/kg lung wt showing weak perinuclear staining of ATII cell (*arrow*). **d** 12-h PE anti-mouse Ccl3 probed lungs exposed to 40 ng curdlan/kg lung wt showing weak perinuclear staining of AM with ingested material (*arrow*). **e** 12-h PE anti-mouse IL1α probed lungs exposed to 40 ng curdlan/kg lung wt showing weak perinuclear staining of AM (*arrow*). **f** 12-h PE anti-mouse Tnf-α probed lungs exposed to 40 ng curdlan/kg lung wt showing conspicious perinuclear staining of AM (*arrow*). (as = alveolar space; aw = alveolar wall). *Bar scales* = 15 μm

mouse lung (Reid et al. 2004). However, unlike results of the Reid et al. study that showed dectin-1 expression confined to alveolar macrophages (AMs) in LPS-injected mice, the present experiments using mice exposed by lung by curdlan instillation demonstrated dectin-1 localization on AMs, alveolar type II cells (ATIIs), and non-ciliated, respiratory bronchiolar epithelia. Dectin-1 transcription and expression on AMs was expected as previous studies have shown that β-glucan- containing fungi can stimulate the dectin-1 receptor on AMs leading to phagocytosis and cytokine production (Steele et al. 2005; Taylor et al. 2006) and that curdlan is a known ligand for dectin-1 in mice (Ferwerda et al. 2008). However, detection of dectin-1 on non-ciliated, respiratory bronchiolar epithelia and in alveolar

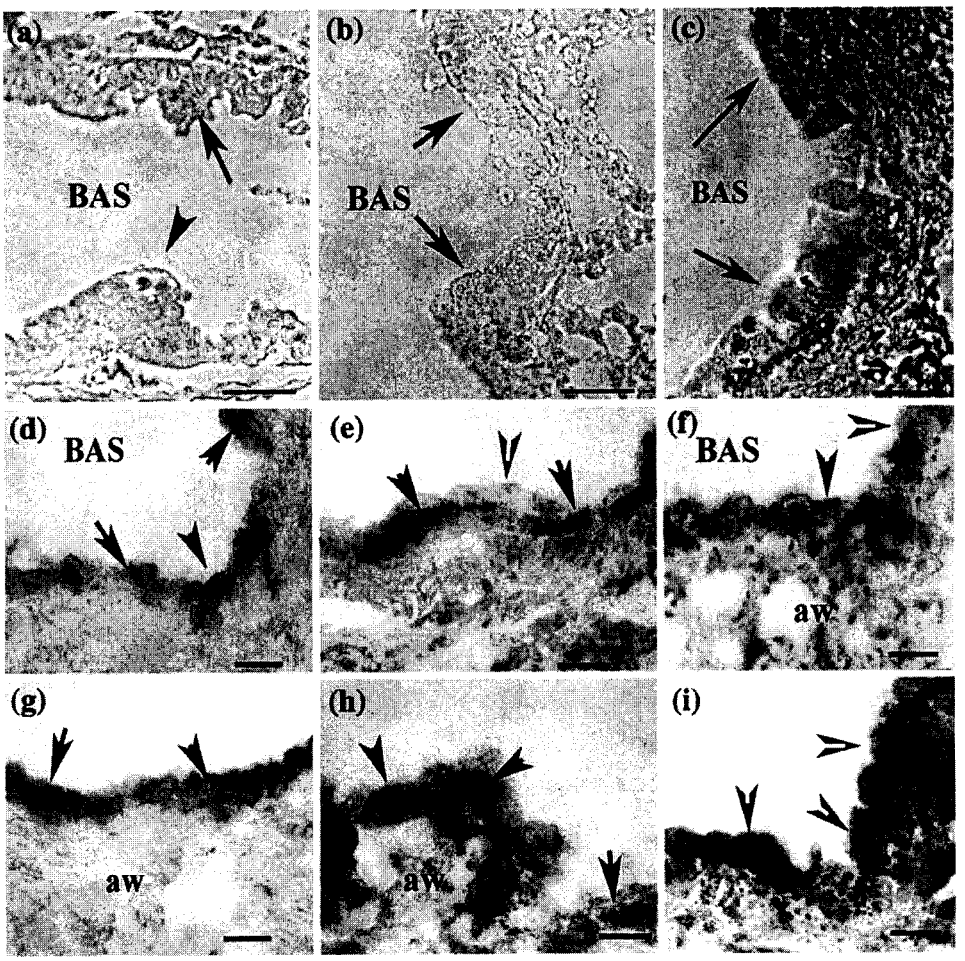

Fig. 5 **a** Immunohistochemical staining of 12-h PE carrier control mouse lung stained with anti-mouse Ccl3 antibody showing an apparent lack of positive staining along bronchiolar epithelium (*arrows*). **b** 12-h PE carrier control mouse lung stained with anti-mouse IL1α antibody showing an apparent lack of positive immunohistochemical staining along bronchiolar epithelium (*arrows*). **c** 12-h PE carrier control mouse lung stained with anti-mouse TNF-α antibody showing inapparent to slight positive immunohistochemical staining along bronchiolar epithelium (*arrows*). **d** 4-h PE anti-mouse Ccl3 probed lungs exposed to 40 ng curdlan/kg lung wt showing weak positive staining of medial and apical bronchiolar epithelium (*arrows*). **e** 4-h PE anti-mouse IL1α probed lungs exposed to 40 ng curdlan/kg lung wt showing weak positive staining of medial and apical bronchiolar epithelium (*arrows*). **f** 4-h PE anti-mouse Tnf-α probed lungs exposed to 40 ng curdlan/kg lung wt showing weak positive staining of medial and apical bronchiolar epithelium (*arrows*). **g** 12-h PE anti-mouse Ccl3 probed lungs exposed to 40 ng curdlan/kg lung wt showing weak positive staining of medial and apical bronchiolar epithelium (*arrows*). **h** 12-h PE anti-mouse IL1-α probed lungs exposed to 40 ng curdlan/kg lung wt showing conspicuous positive staining of medial and apical bronchiolar epithelium (*arrows*). **i** 12-h PE anti-mouse Tnf-α probed mouse lung exposed to 40 ng curdlan/kg lung wt showing conspicuous positive staining. (aw = alveolar wall; BAS = bronchio-alveolar space). *Bar scales* = 20 μm

type II cells (ATIIs) is a new finding. Localization of dectin-1 mRNA transcript and expression along the respiratory bronchiolar epithelia further confirms the important immunomodulatory function of these cells (Kelly et al. 2008) and their potential role in the recognition of, and response to fungal glucan (Allen et al. 2001). Additionally, *dectin-1* localization on ATIIs using ISH and IHC indicates that these cells, like AMs, may be major dectin-1 expressing cells lining the alveoli. While ATIIs are known to express toll-like receptors and play an important role in the innate immunity of the lungs (Torrelles et al. 2008), as far as we are aware, their ability to recognize glucan is unknown and deserves further study.

Using RT–PCR array technology, our results also showed that curdlan exposures induced dose- and time-dependent transcription of inflammation-associated genes in mice intratracheally exposed to a single dose of curdlan at concentrations ranging from 4 μg to 4 ng curdlan/kg lung wt. That these transcriptional changes were in lung tissues stripped of the major respiratory airways suggest that these responses were mediated by the stimulation of respiratory bronchiolar and alveolar surfaces where the majority of

inhaled glucan is likely to be efficiently deposited (Kleinstreuer et al. 2007, 2008; Phalen et al. 2008). Combined with the ISH and IHC experiments, these results provide in situ evidence that exposures of (1-3)-β-D glucan in a triple helical form can potentiate non-allergenic pro-inflammatory responses by stimulating the glucan receptor dectin-1 on the surfaces of immunosentinel cells in the lower respiratory tract.

The dose-dependent transcriptional responses were manifested in the number of gene transcripts significantly modulated in animals exposed to 4–0.4 μg curdlan/kg lung wt (54 vs. 40 genes expressed) compared to those exposed to 40–4 ng curdlan/kg lung wt (21 vs. 9 genes expressed) and generally in the significantly higher gene transcription levels at the highest (4–0.4 μg curdlan/kg lung wt) compared to the lowest doses (40–4 ng curdlan/kg lung wt). The up-regulation of higher numbers of genes and significantly higher transcription levels at the highest curdlan concentrations helps provide a mechanistic basis for the dose-dependent leukocyte responses in guinea pig BALF (Fogelmark et al. 1997) and histopathological responses in curdlan-instilled mice reported by Schuyler et al. (1998). Of the gene transcripts significantly expressed, 21 were modulated from 4 μg to 40 ng curdlan/kg lung wt while only 9 were also expressed at 4 ng curdlan/kg lung wt treatment suggesting that these 9 genes may serve central roles in the modulation of the inflammatory responses to this glucan. The majority of these 9 genes were ones associated with acute pulmonary inflammation (e.g., *Ccl3, Ccl11* (=*eotaxin*), *Ccl17, Il1α, Il20, Tnf-α, Cd40lg*), especially in mediating acute inflammatory recruitment response of eosinophils (*Ccl11, Ccl3*), AM's (*Il1a, TNF-α*), neutrophils (*Ccl17*), macrophages (*Ccl3, Il1a, Tnf-α*), T-cells (*Ccl17, Ifng*), and bronchiolar epithelium remodeling (*Tnf-α*), among others (Didierlaurent et al. 2006; Doucette et al. 2002; Elizur et al. 2008; Kaufman et al. 2004; Kelsen et al. 2004; Huaux et al. 2005; Hernandez-Novoa et al. 2008; Ishida et al. 2007; Murphy et al. 2008; Ohkawara et al. 2005; Pease and Sabroe 2002; Suzaki et al. 2008). *Cd40lg* is a key signaling conduit involved in the activation of non-haematopoietic cells, such as fibroblasts, and endothelial cells leading to their production of pro-inflammatory mediators (Kaufman et al. 2004). While it is unclear from the present study whether all these cell types were responding to curdlan exposure, results of the IHC experiments were insightful. These experiments not only confirmed gene array data by showing that three of the genes (*Ccl3, Il1α,* and *Tnf-α*) identified as having central roles in the modulation of the curdlan-induced inflammatory responses were co-expressed in mice exposed to at least 40 ng curdlan/kg lung wt, but that expression of these genes was associated with respiratory bronchiolar epithelium, ATIIs, and AMs. As these were the same cells that transcribed and expressed dectin-1, these results further highlight the important role they appear to have in the innate immune orchestration of pro-inflammatory cytokine release and immunomodulation of lungs exposed to curdlan.

Up-regulation of *Il1α* was an interesting observation. This gene has been implicated in fever onset (Murphy et al. 2008) and bio-behavioral modulation in mice (Furuzawa et al. 2002). The latter supports our suggestion that exposure to fungal glucan may form a partial basis for the claim that mold exposures result in neurocognitive outcomes in exposed populations (Miller et al. 2003). This should be explored in further animal studies.

While the majority of gene transcripts were significantly up-regulated, several (*Ccr3, Cxcl13*, and *Il6st*) transcribed at the two highest exposure levels only were significantly down-regulated in curdlan-instilled lungs. These genes are important in the accumulation and trafficking of neutrophils and eosinophils, among others (Lloyd 2002; Murphy et al. 2008). For example, *Ccr3* binds and responds to a variety of chemokines, including *Ccl5, Ccl11, Ccl13*, and *Ccl17*, among others. *Ccr3* is highly expressed in eosinophils and basophils and is also detected in TH1 and TH2 cells, as well as in airway epithelial cells (Murphy et al. 2008). This receptor may contribute to the accumulation and activation of eosinophils and other inflammatory cells in the allergic airway. Thus, down-regulation of these genes in the treatments exposed to highest but not in the lowest curdlan doses may help explain the results of Fogelmark et al. (1992, 1997) that showed depressed leukocyte influx in lungs of guinea pigs exposed to from 0.5 to 25 μg/ml curdlan. Moreover, such dose-dependent patterns of gene transcription and expression may also provide a mechanistic basis for differences in outcome between Sigsgaard et al. (2000) and Bonlokke et al. (2006) studies and those of Rylander (1993, 1996) which indicated that the direction of health effects from glucan varied in direction depending on exposure and effect measured.

While only two experimental end points were used in the present study, time-dependent gene transcription was evident for 8 genes in treatment animals at 4 h but not at 12-h PE, and 12 gene transcripts that were expressed at 12 h but not at 4-h PE. That IHC using anti-mouse Ccl3, IL1a, and *Tnf-α* antibodies revealed that staining of lungs exposed to 40 ng curdlan/kg lung wt was more intense at 12-h PE than at 4-h PE lends further support for the transcriptional time-dependency results revealed by the array assays, especially as protein expression lags transcription. These results not only suggest that lung response to curdlan is rapid (within 4-h PE), which supports the ISH and IHC experiments, they also support results of the study by Kataoka et al. (2002) that showed rapid (within 2-h PE) iNos, Tnf-α, and MIP-2 expression in RAW-264.7 and RAW-R12 macrophage lines exposed to curdlan. They also support results of

previous studies that showed temporality as well as acute (within 4-h PE) lung responses in mice exposed to low-molecular weight fungal compounds (Rand et al. 2005, 2006). It is unclear why there were temporal transcription patterns or why the majority of these significantly modulated genes at these two time points were receptors (5/8 genes at 4 h and 7/13 genes at 12-h PE). However, it may reflect the complexity of cytokine immunology that involves a cascade of molecular events from the activation of receptors and their signal transduction pathways to the production of inflammatory mediators, including pro-inflammatory cytokines and chemokines, adhesion molecules, reactive oxygen species such as nitric oxide by various cell types of the lungs (Jeyaseelan et al. 2004). Differential time course in expression of inflammation-associated genes has been reported as a response in LPS-induced acute lung injury (Jeyaseelan et al. 2004). These investigators attributed this temporal pattern to the differential recruitment of cells into lungs as well as an increase in transcription mechanisms of various cell types in the lungs, and may also explain our findings.

In summary, dose and time-dependent transcription and expression of dectin-1 and inflammation-associated genes were observed in mice intratracheally exposed to 4 μg to 4 ng curdlan/kg lung wt. Interestingly, this is close to the minimal amount of curdlan that activates the *Limulus* glucan receptor (Tanaka et al. 1991). It is also within the range of airborne mold glucan concentrations in which occupant symptoms have been reported (Rylander and Lin 2000). One isoform of the human glucan receptor is similar to its murine homologue (Willment et al. 2001). These observations provide a biological basis for the irritant and inflammatory response to curdlan observed in humans in experimental studies (Bonlokke et al. 2006; Rylander 1993, 1996; Sigsgaard et al. 2000).

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